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TITLE: Development of Biodegradable Zinc Oxide Nanowires Targeting Breast Cancer Metastasis

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**14. ABSTRACT**

Our objective is to develop a biodegradable ZnO nanomaterial platform (mainly focusing on the nanowire [NW] morphology) for efficient vasculature targeting of BCa metastasis, and to optimize its optical property and conjugation chemistry for non-invasive dual-modality tracking (PET and optical). The overarching hypothesis is that suitably functionalized ZnO NWs can have long circulation lifetime and efficient tumor targeting for future drug delivery applications in treating BCa metastasis.

Specific Aims:

Aim 1: To develop strongly fluorescent ZnO NWs with desired size and emission wavelength.

Aim 2: To optimize the biocompatibility and integrin αvβ3 targeting efficacy of ZnO NWs in vitro.

Aim 3: To optimize the BCa metastasis targeting efficacy of ZnO NWs in vivo.

**15. SUBJECT TERMS**- none provided

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**DOD BCRP BC103896: W81XWH-11-1-0648**
**PI: Yunan Yang, MD, Ph.D, University of Wisconsin-Madison**
**Supplement to the 2nd annual technical report to address the following comments:**

*"Not Scientifically acceptable in accordance to the contract Statement of Work. Return report to Principal Investigator for rewrite with an UNLIMITED distribution (Approved for Public Release; Distribution Unlimited)*

*Comments to PI - Please revise your report to address the progress you made against the statement of work tasks.*

*Task 1. To develop strong fluorescent ZnO NWs with desired size and emission wavelength (months 1-24; Yang). Not addressed.*
*Task 2. To optimize the biocompatibility and integrin alphavbeta3 targeting efficacy of ZnO NWs in vitro (months 1-24; Cai). Not addressed.*
*Task 3. To optimize the BCa metastasis targeting efficacy of ZnO NWs in vivo (months 13-36; Cai). Not addressed."*
*Task 4. Explore other ideas in breast cancer research to carry over to independent translational research (months 13-36). Not addressed."*

**Task 1 and Task 2:**

As described in the last fellowship report most of the work concerning Tasks 1 and 2 were accomplished during the 2012-2013 period. The following list summarizes the major research accomplishments in this regard:

1. We successfully generated a strongly-luminescent ZnO nanoplatform (NP).
2. The bioconjugation of ZnO NP to a receptor targeting ligand was achieved.
3. The evaluation of ZnO NP *in vivo* toxicity was conducted for the first time.
4. We optimized some of the physical properties of ZnO NP such as particle size; **Figure 1** shows the ZnO NP size and surface properties by SEM.
5. Based on our previous studies, CD105 was established as an advantageous angiogenesis biomarker compared with integrin $\alpha_v\beta_3$, both *in vitro* and *in vivo*. The evidence presented in **Figure 2** attest for the excellent targeting efficacy of ZnO NP labeled with TRC105, an anti-CD105 monoclonal antibody that has high affinity for CD105 expressed endothelial cells on breast cancer (BC).

**Task 3:**

*In vivo* studies on breast cancer xenograft in mice were completed in accordance with the objective delineated in Task 3 (**Figure 3-6**). However, the metastasis models were under

development. Breast cancer, lung and brain metastasis tumor models are projected to be executed and finished within the next reporting period.

**Task 4:**

As part of our efforts to develop new strategies for diagnostic and therapy of BC, we developed and anti-CD146 monoclonal antibody (named YY146) to target the highly expressed CD146 epitope in triple-negative BC. The production of our antibody was carried out using a novel antibody production strategy that enabled fast an efficient antibody production. Some of the most relevant advances using YY146 are summarized in the following list:

1. Successful generation of highly-specificity anti-CD146 monoclonal antibody.
2. Bioconjugation of YY146 to a chelating moiety (NOTA) for the radiolabeling with $^{64}Cu$ and subsequent non-invasive PET imaging of a brain tumor model was accomplished. Our next step is to try YY146 for imaging of several BC models.
3. Mechanism exploration on YY146 interaction with its antigen was undertaken, showing the potential of this antibody as a therapeutical alternative.
4. Elevated CD146 expression was observed in triple-negative breast cancer cell line MB-231 (**Figure 7**) which most certainly indicates the potential applicability of therapeutic and diagnostic strategies using YY146.
5. Triple-negative breast cancer cell line MB-231 can be targeted by ZnO-PEG-YY146-FITC as depicted in **Figure 8**. More optimization in the nanoparticle properties and bioconjugation are under way before we can move on to in vivo testing.
6. Finally, our preliminary results indicate that the metastastic capacity of triple-negative breast cancer cell line MB-231 can be inhibited by treatment of the cells with YY146 (**Figure 9**).

Triple-negative breast cancer (TNBC) is an aggressive subtype that is highly associated with poor prognosis. Even after first line treatment with surgery and chemotherapy, a large fraction of the tumors recur; this recurrence often resulted from improper tumor resection and the existence of undetectable metastatic loci. Many of these difficulties with management of TNBC could be attribute to pathologic chemo-resistance. An increasing number of reports have described new targets against many different TNBC biomarkers associated with chemo-resistance and metastasis [1]. So there is a great potential in the targeting of these aberrantly

expressed markers which might lead to new treatment strategies that might improve quality of life for patients.

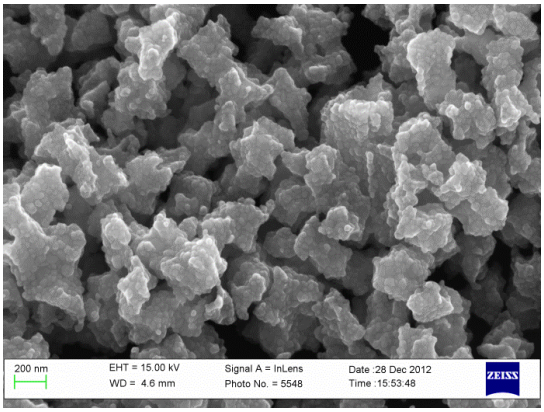
Nanotechnology holds tremendous potential for early detection, accurate diagnosis, and personalized treatment of cancer. Herein we report the synthesis of green/red fluorescent ZnO nanoplatforms (including both NWs and NPs) and demonstrated as a proof-of-principle that ZnO NPs can be functionalized to specifically target breast cancer cell surface receptors *in vitro*. As a consequence the use of ZnO NPs, which can be loaded with significant amount of anticancer drugs, may open new avenues of future research in tumor-targeted drug delivery [2]. The one-dimensional shape of a ZnO NP is highly desirable for efficient tumor targeting since such morphology can readily take advantage of the polyvalency effect. After optimizing the conjugation time, PH value, temperature et al, we finally obtained ZnO NP with desired size and morphology; **Figure 1** shows the morphology (by SEM) and luminescent behavior of ZnO NP.

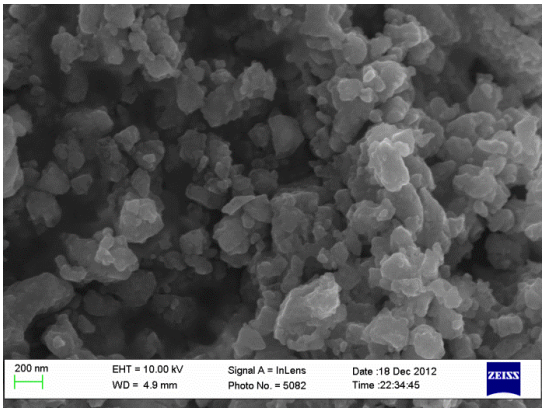
After extensive testing and based on our body of data, we determined that CD105 is a more efficient angiogenesis target than integrin $\alpha_v\beta_3$, both *in vitro* and *in vivo*. **Figures 2** and **3** show the ability of ZnO NPs labeled with TRC105, anti-CD105 monoclonal antibody, to determine the expression of CD105 in cells as well as BC xenografts. Positron emission tomography (PET) evidenced a specific elevated accumulation of 15 %ID/g in xenografts BC tumors, which demonstrates a highly efficacy of CD105 targeting (**Figure 2- 6**).

Nevertheless, CD105 is a target that is preferentially expressed within tumor neo-vasculature (a well-known angiogenesis marker), being its expression in tumor cell line is often low. Many commercially available monoclonal antibodies, as well as those that are under development, target angiogenesis[3]. Because of that and considering the highly aggressive character of triple negative breast cancer, we are looking for a specific maker to target the BC cells directly [4]. After a comprehensive review of recent literature, we noticed that patients diagnosed with late stage of TNBC, typically show significantly higher levels of CD146, compared to demographically matched healthy people [5-7]. These findings evidence the vital role that CD146 plays in tumor progression, aggressiveness, and metastasis [8]. Hence, we believe CD146 can be a good target for triple-negative breast cancer [9-11]. In order to get a high affinity, sensitive, and specific monoclonal antibody, we devised a novel method of mouse immunization which allowed us to obtain activated B-cells within a two week time frame [12]. After the antibody screening process, we obtained five clones of anti-CD146 monoclonal

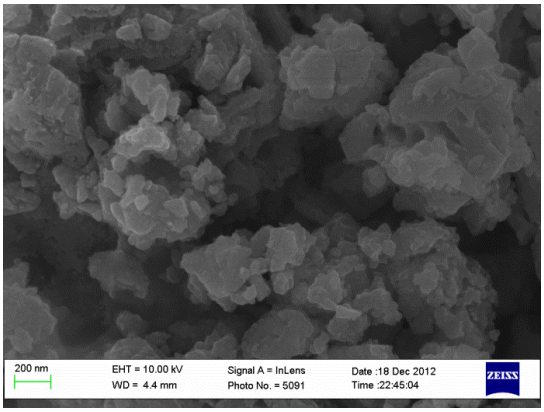
hybridoma cell lines and named the best antibody clone as YY146. YY146 successfully recognizes the CD146 epitope in a plethora of aggressive cancer cells *in vitro* including triple-negative breast cancer, glioma, gastric cancer, colon cancer, and cervical cancer, etc. In Task 4 we stated that we would explore other ideas in breast cancer research that would carry over into independent translational research. We are committed to complement our current results (Figures 7-10) by further developing and testing our antibody *in vivo*. We can then assess its potential as a new diagnostic and therapeutic strategy to combat the extremely aggressive TNB that is associated with poor prognosis.

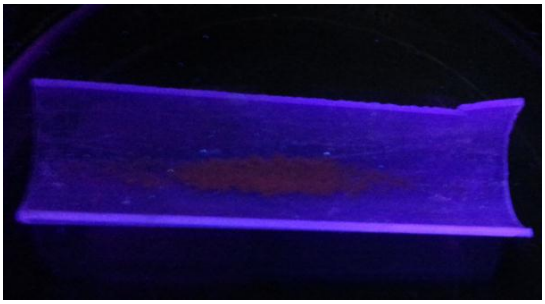
**Figures (Total of 10)**




ZnO NP NOTA-ZnO NP-PEG



NOTA-ZnO NP-PEG-Ab

**Figure 1.** Charaterization of ZnO NP. ZnO NP shows a stable size, morphology before and post antibody labeling (by SEM) and luminescent behavior of ZnO NP (a photograph of ZnO NP). SEM scale bar: 200 nm.

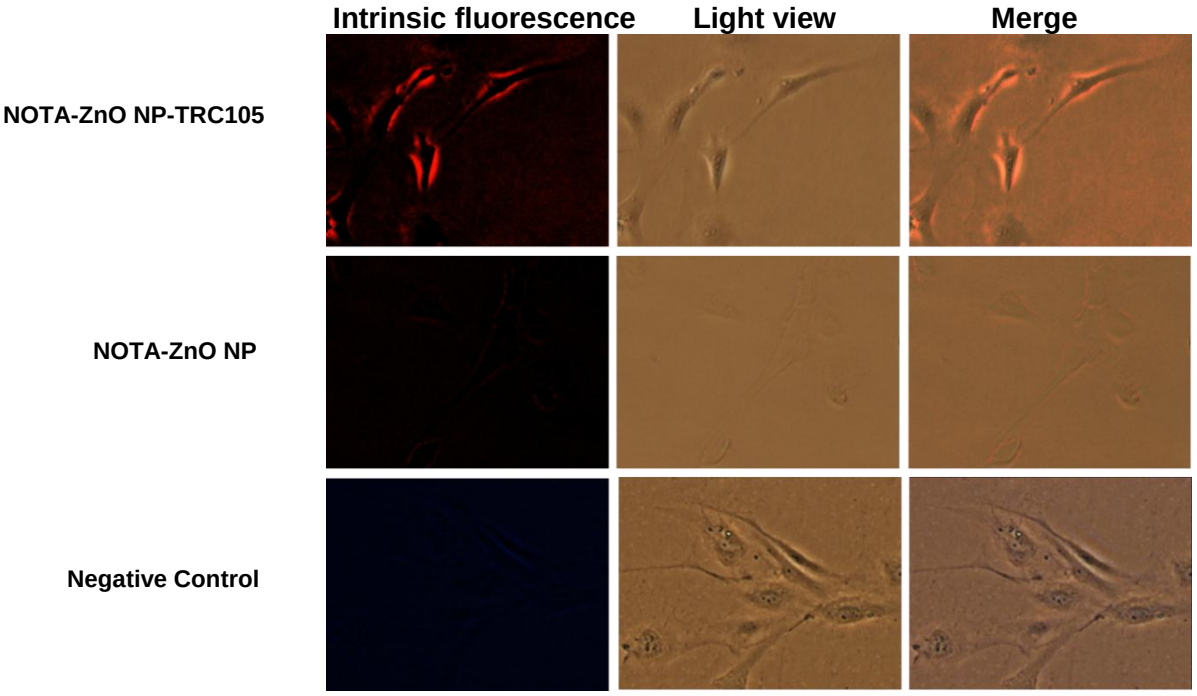



**Figure 2.** Fluorescence imaging of CD105 expression on fixed human endothelial cells with NOTA-ZnO NP and its conjugates NOTA-ZnO NP-TRC105. A concentration of 30 μg/mL (based on ZnO NP) and an incubation period of 1 h were adopted. All fluorescence images were acquired under the same condition and displayed using the same scale. Magnification: 200x.

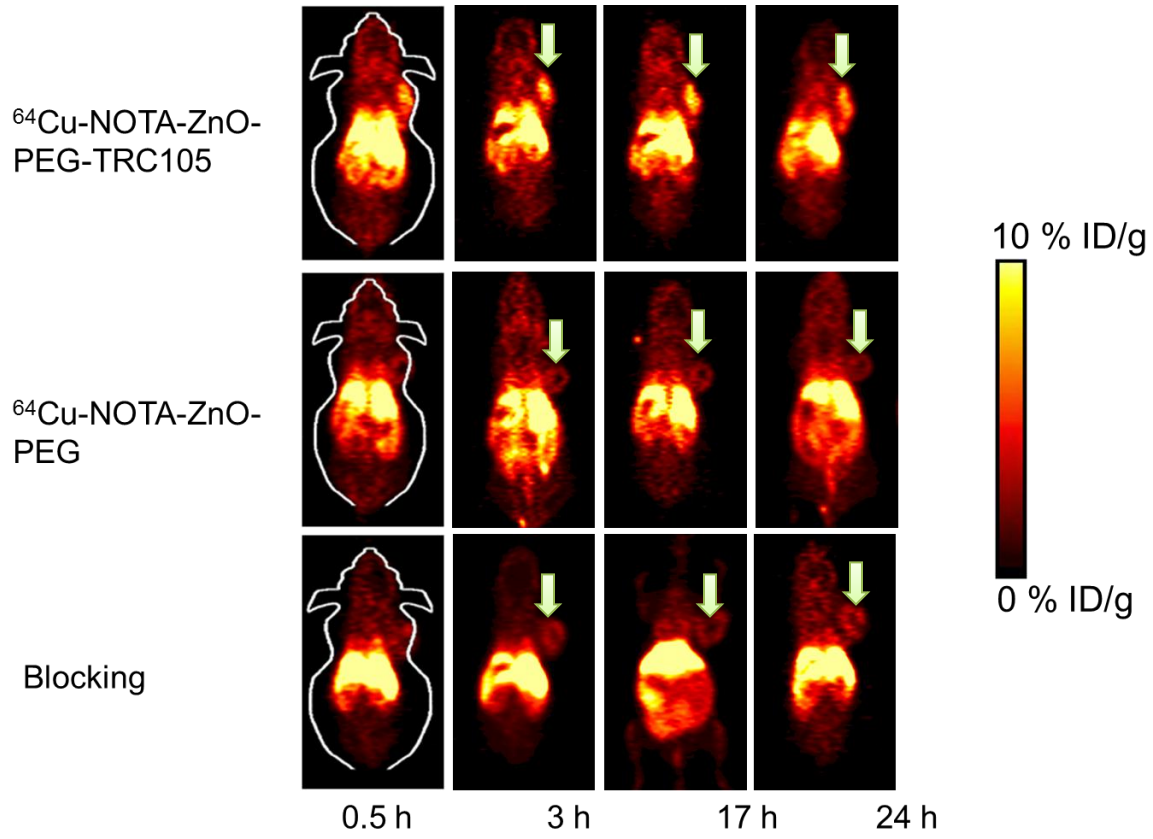



**Figure 3.** In vivo investigation of $^{64}$Cu-NOTA-ZnO-PEG-TRC105 in 4T1 tumor bearing mice. A serial of coronal PET images about 4T1 tumor-bearing mice at 0.5, 3, 17 and 24 post-injection of $^{64}$Cu-NOTA-ZnO-PEG-TRC105, $^{64}$Cu-NOTA-ZnO-PEG and TRC105 before $^{64}$Cu-NOTA-ZnO-PEG-TRC105 (i.e., blocking). Tumors are indicated by arrowheads.

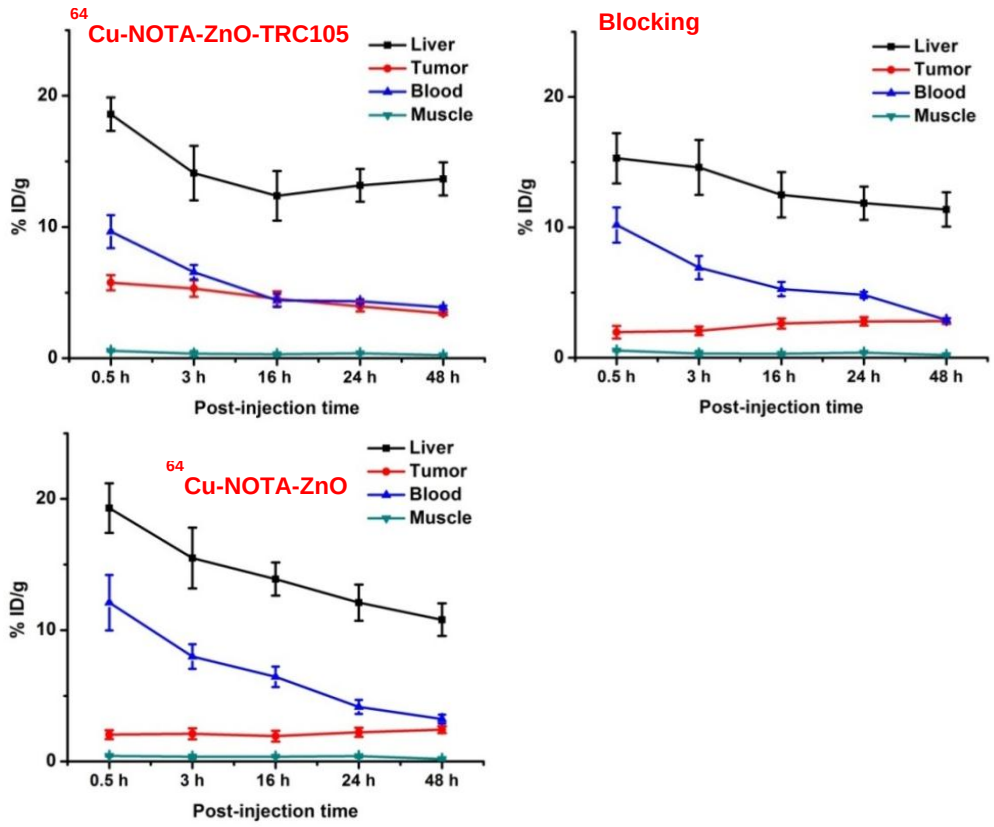



**Figure 4.** Comparison of 4T1 tumor uptake of $^{64}$Cu-NOTA-ZnO-PEG-TRC105, $^{64}$Cu-NOTA-ZnO-PEG-TRC105 with a blocking dose of TRC105, and $^{64}$Cu-NOTA-ZnO-PEG. Time-activity curves of the tumor, liver, blood, and muscle upon intravenous injection $^{64}$Cu-NOTA-ZnO-PEG-TRC105 into 4T1 tumor-bearing mice (n=3). *p<0.05 (n=3)

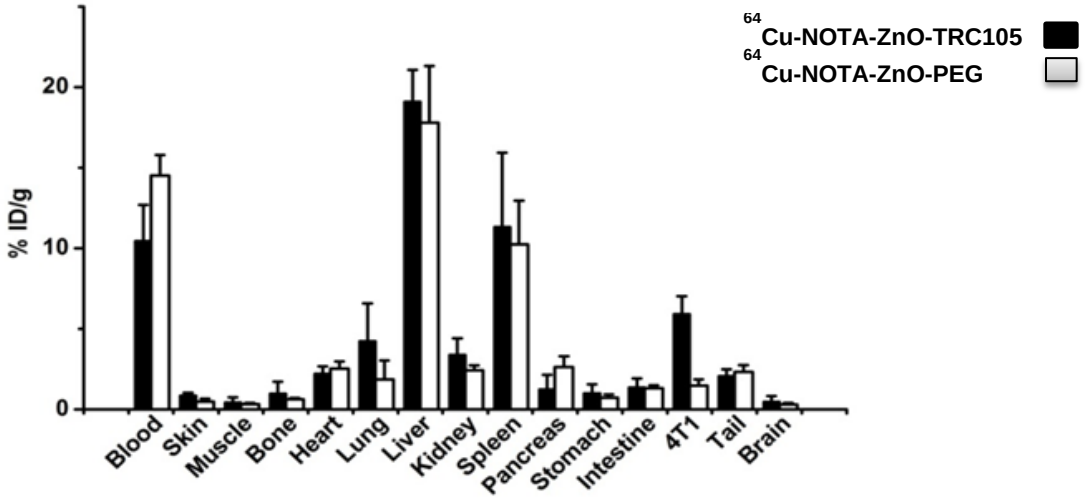



**Figure 5.** Biodistribution studies after noninvasive PET scans. A Biodistribution of $^{64}$Cu-NOTA-ZnO-PEG-TRC105 and $^{64}$Cu-NOTA-ZnO-PEG in 4T1 tumor-bearing mice at 5h postinjection (n=3). Besides the liver and spleen, the blood also had significant tracer uptake at 5 h p.i. *$p<0.05$.

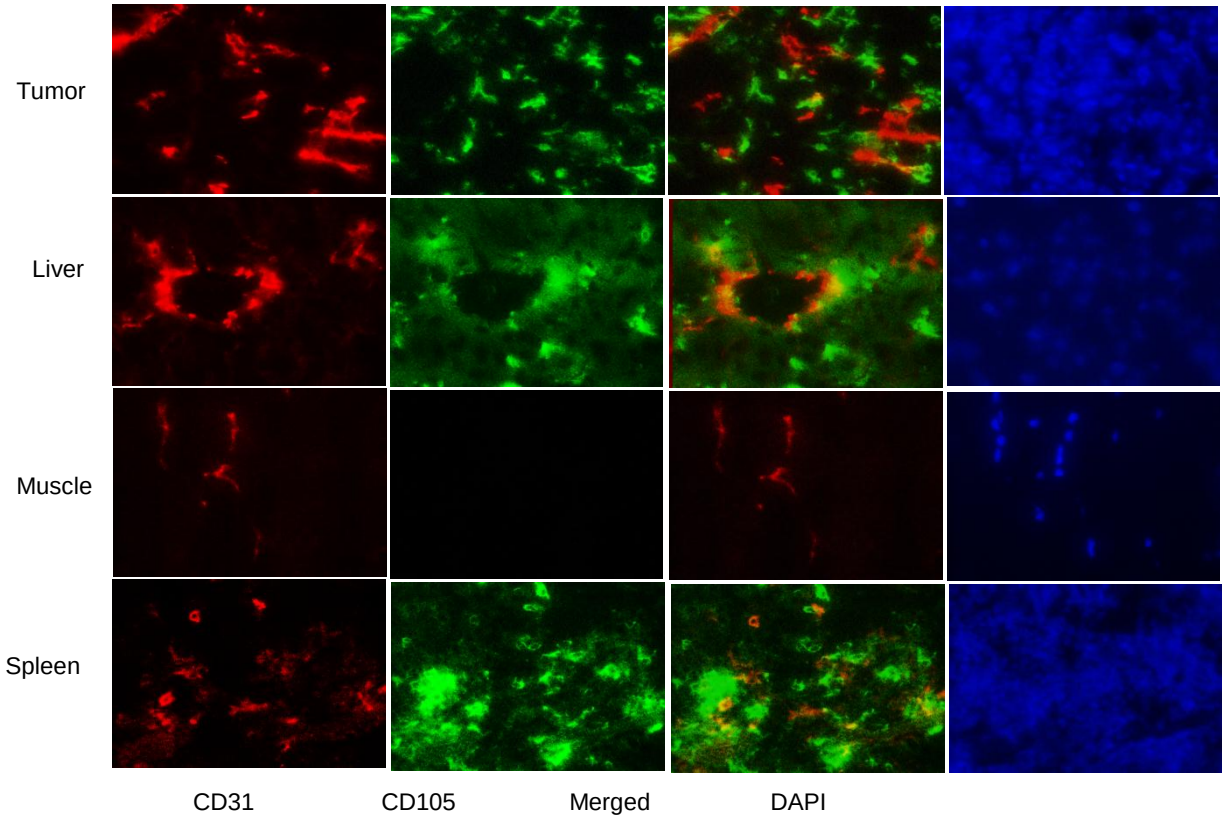



**Figure 6.** Immunofluorescence CD105/CD31 double staining of the 4T1 tumor, liver, muscle and spleen tissue sections. TRC105 and AlexaFluor488-labeled goat anti-human IgG was used for CD105 staining (green). The tissue slices were stained with rat anti-mouse CD31 antibody and Cy3-labeled donkey anti-rat IgG (red). Immunofluorescence CD105/CD31 staining of various tissues ex vivo revealed that CD105 expression in the 4T1 tumor was primarily on the tumor vasculature, as evidenced by excellent colocalization of CD105 and CD31 staining and very weak signal on the 4T1 tumor cells. CD105 staining of mouse liver and spleen both gave very low signal, indicating that these tissues do not have a significant level of CD105 expression. Therefore, tracer uptake in the liver and spleen was largely unrelated to CD105 binding and more likely related to nonspecific capture by the reticuloendothelial system, hepatic clearance.

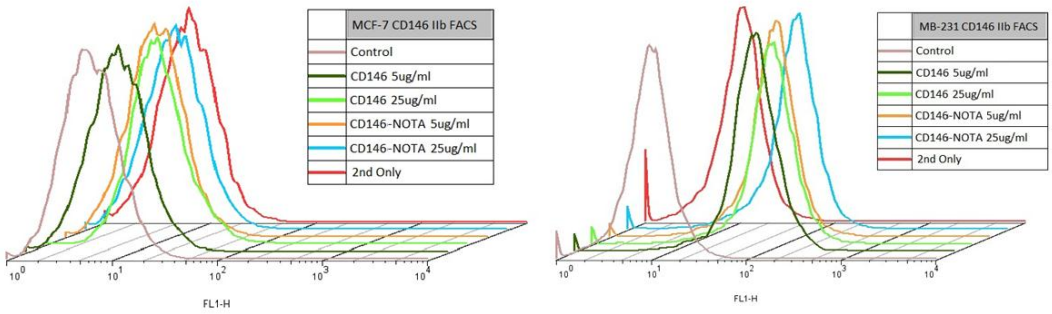



**Figure 7.** Flow cytometry analysis in both MCF-7 (CD146-negative) and MB-231 (CD146-positive) cells exhibited no detectable differences in specificity and affinity between YY146 and NOTA-YY146 with two concentrations used in this study (5 or 25μg/mL).

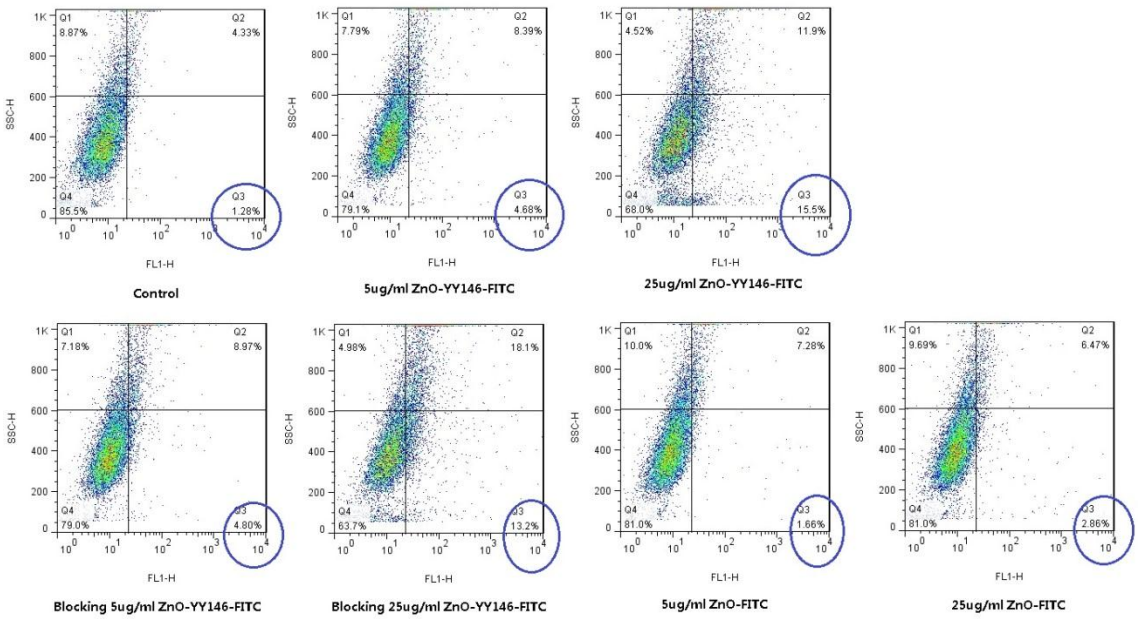



**Figure 8.** Comparison of MB-231 (triple-negative breast cancer cells) uptake of ZnO-YY146-FITC and ZnO -FITC, with a blocking dose of YY146 and ZnO-YY146-FITC. We used two concentration in this study (5 or 25 ug/ml). There is a significant difference between ZnO-YY146-FITC (15.5%)and ZnO –FITC (2.86)group but minor difference with blocking group (13.2%). We will increase the blocking time from 10 into 30 min confirm the specificity of YY146 on MB231.

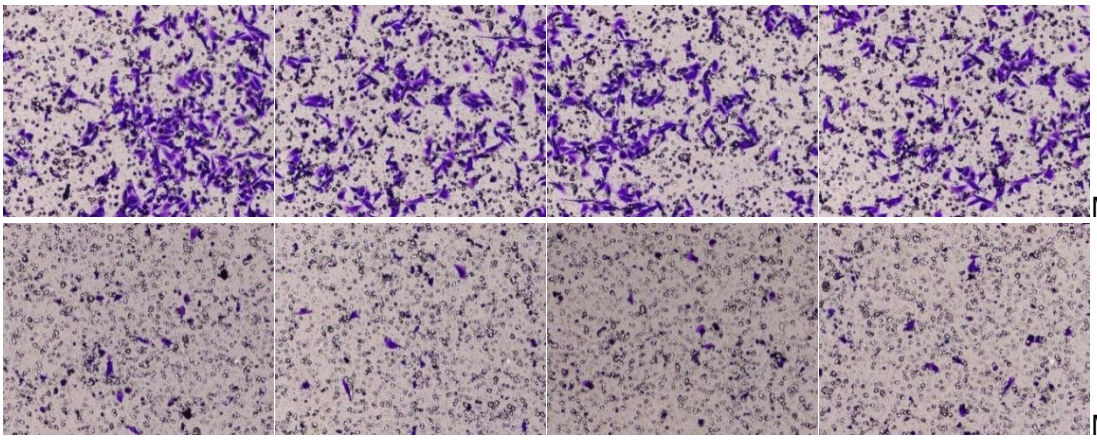



**Figure 9.** The impact of YY146 on the invasive property of MB-231 was evaluated by Matrigel with a Boyden chamber assay. Cells were incubated with 10 μg/ml YY146 were significantly less invasive than cells without treatment (Mock).

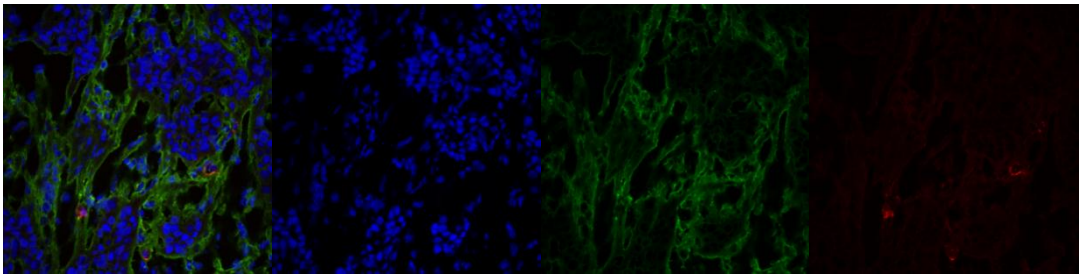



**Figure 10.** Confocal Fluorescence microscope of CD146 on MB-231 tumor tissue Immunofluorescence CD146/CD31 staining of xenograft breast tumor tissue ex vivo revealed that CD146 expression in the breast cancer was primarily on the tumor membrane.

## References


1. Martin, H.L., L. Smith, and D.C. Tomlinson, *Multidrug-resistant breast cancer: current perspectives.* Breast Cancer (Dove Med Press), 2014. **6**: p. 1-13.
2. Xu, Y., et al., *Detection and identification of breast cancer volatile organic compounds biomarkers using highly-sensitive single nanowire array on a chip.* J Biomed Nanotechnol, 2013. **9**(7): p. 1164-72.
3. Barton Md, M.K., *Bevacizumab in neoadjuvant chemotherapy increases the pathological complete response rate in patients with triple-negative breast cancer.* CA Cancer J Clin, 2014.
4. Danova, M., et al., *Tissue and soluble biomarkers in breast cancer and their applications: ready to use?* J Natl Cancer Inst Monogr, 2011. **2011**(43): p. 75-8.
5. Choi, Y., et al., *Epithelial-mesenchymal transition increases during the progression of in situ to invasive basal-like breast cancer.* Hum Pathol, 2013. **44**(11): p. 2581-9.
6. Imbert, A.M., et al., *CD146 expression in human breast cancer cell lines induces phenotypic and functional changes observed in Epithelial to Mesenchymal Transition.* PLoS One, 2012. **7**(8): p. e43752.
7. Zeng, G., et al., *METCAM/MUC18 augments migration, invasion, and tumorigenicity of human breast cancer SK-BR-3 cells.* Gene, 2012. **492**(1): p. 229-38.
8. Zeng, Q., et al., *CD146, an epithelial-mesenchymal transition inducer, is associated with triple-negative breast cancer.* Proc Natl Acad Sci U S A, 2012. **109**(4): p. 1127-32.
9. Zeng, G.F., S.X. Cai, and G.J. Wu, *Up-regulation of METCAM/MUC18 promotes motility, invasion, and tumorigenesis of human breast cancer cells.* BMC Cancer, 2011. **11**: p. 113.
10. Mostert, B., et al., *Detection of circulating tumor cells in breast cancer may improve through enrichment with anti-CD146.* Breast Cancer Res Treat, 2011. **127**(1): p. 33-41.
11. Zabouo, G., et al., *CD146 expression is associated with a poor prognosis in human breast tumors and with enhanced motility in breast cancer cell lines.* Breast Cancer Res, 2009. **11**(1): p. R1.
12. Grivel, J.C., et al., *Rapid induction of anti-idiotypic responses to unmodified monoclonal antibodies from syngeneic mice following primary immunization.* J Immunol Methods, 1993. **158**(2): p. 173-82.